

Roles of *Averrhoa bilimbi* Extract in Increasing Serum Nitric Oxide Concentration and Vascular Dilatation of Ethanol-Induced Hypertensive Rats

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ABSTRACT: The considerably high incidence of cardiovascular disease in Indonesia has attracted scientists to investigate various plant and fruit extracts as preventive agents. *Averrhoa bilimbi* (AB) is rich in bioactive constituents that may be effective in preventing indicators of hypertension. This study evaluated the roles of AB extract in increasing serum nitric oxide (NO) concentration and vascular dilatation in ethanol-induced hypertensive rats. A total of 24 male Wistar rats (*Rattus norvegicus*) were divided equally into 4 treatment groups (n=6): P0 (control group, administered placebo); P1 [administered captopril 3 mg/kg body weight (BW) orally]; P2 (administered AB extract at 20 g/kg BW); and P3 (administered AB extract at 40 g/kg BW). The AB extract was obtained from fresh AB macerated in 96% ethanol and was subjected to bioactive compounds identification using thin layer chromatography. After pretreatment with ethanol for 15 days, treatments were administered daily for 14 days. All rats were measured for tail blood pressure by the tail-cuff method and NO concentrations by avidin-horseradish peroxidase sandwich-enzyme-linked immunosorbent assays. All rats were sacrificed to collect blood vessels for histopathology. The results showed that AB extracts contained flavonoids, saponins, polyphenols, essential oils, and anthraquinone. Treatment with AB extract at a dose of 40 mg/kg BW significantly increased NO concentrations ($P < 0.05$). Histopathological analysis showed that AB extracts inhibited endothelial pyknosis, intimal body, and adventitial leukocyte infiltration of posterior vena cava blood vessels. These results suggest that the protective effect of AB extracts is associated with NO concentration in the blood by inhibiting blood vessel dysfunction.

Keywords: antioxidants, blood pressure, cardiovascular disease, hypertension, rats

INTRODUCTION

Oxidative stress is a physiological condition that arises from lack of an antioxidative defense system to balance excessive reactive oxygen species (ROS) production, which may cause several health problems and diseases (Rezaie et al., 2007). Antioxidant defense systems, including endogenous, exogenous, and non-enzymatic compounds, play a crucial role as effective scavengers to limit ROS production and ultimately reduce the risk of diseases related to oxidative stress, including hypertension, polycystic ovarian syndrome (PCOS), and other inflammatory diseases (Heshmati et al., 2020; Morvaridzadeh et al., 2020).

Hypertension is one of the most prevalent disease, but is often undiagnosed and unmanaged until people get their blood pressure (BP) measure. Hypertension can affect all age groups in all socio-economic levels (Bailey et al., 2010). Research on experimental animals has shown that an increase in BP is associated with an increase in oxidative stress. Increased oxidative stress promotes vascular smooth muscle cell proliferation and hypertrophy and collagen deposition, causing thickening of the vascular media and narrowing of blood vessel lumens. This can damage the endothelium and impair endothelium-dependent relaxation of blood vessels, which increases vascular contractile activity and leads to hypertension (Grosman, 2008).

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The prevalence of hypertension in Indonesia is relatively high due to traditional lifestyles changing to modern lifestyles. The death rate from cardiovascular disease in the elderly with hypertension is three times higher than in those without hypertension. In general, the prevalence of hypertension ranges from 1.80 to 28.6% in adults \geq 20 years of age. However, several studies in Indonesia have shown that the prevalence ranges from 17 to 22%. Peltzer and Pengpid (2018) reported that 33.4% of the Indonesian population has hypertension, including 31.0% of men and 35.4% of women.

Treatment with substances containing antioxidants has been widely studied as a means to reduce oxidative stress and BP. Certain fruits contain active antioxidant compounds that inhibit oxidative reactions in target cells (Materska, 2008). Antioxidant compounds in fruits are often more beneficial compared with synthetic drugs, since they have relatively low toxic effects and do not generally cause allergies (Wang et al., 2009).

Free radicals are chemical compounds that have one or more unpaired electrons, which are unstable and highly reactive. To achieve stability, these electrons must find another electron to couple. The chain reaction of free radical formation leads to formation of more free radicals, known as oxidative stress. The human body is equipped with an antioxidant system that functionally prevents formation of and neutralizes antioxidants. The antioxidant system comprised of the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, and the chain breaker antioxidants vitamins E and C, and beta-carotene. In addition, the antioxidant repair system contains enzymes that repair or remove fat molecules, proteins, or DNA damaged by oxidative reactions (Halliwell, 1997).

During oxidative stress, free radicals, or ROS [including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxylation (OH^-)], and nitrite radicals or reactive nitrogen species (RNS) [including nitroxyl (NO^-) and peroxynitrite ($ONOO^-$)], which is a molecule derive from O_2 , play important roles in the pathophysiology of blood vessels by oxidation/reduction (redox) reactions. Blood vessel cells (including endothelial, smooth muscle, and fibroblasts) can produce free radicals or ROS by oxidation of nicotinamide-adenine dinucleotide phosphate hydrate [NAD(P)H] in cell membranes. Free radicals can interfere with blood vessel function by several mechanisms, including modulation of cell growth, apoptosis, migration, inflammation, secretion, and extracellular matrix protein production (Touyz and Schiffrin, 2004).

Nitric oxide (NO) is pivotal to the function of the body's pathophysiological processes. NO molecules were originally identified from the formation of L-citrulin from L-arginine by its role in mediating NO synthase (NOS) enzymes, one of which is secreted by endothelium-de-

rived relaxing factors. Studies have shown NO plays important roles as a neurotransmitter, vasodilator, and anti-inflammatory mediator (Ricart-Jané et al., 2002; Crawford et al., 2004), as an anti-bacterial and leukocyte activator (Lim et al., 2008), and in gene therapy for cardiovascular disease (Gladwin et al., 2006; Mannick, 2006; Minneci et al., 2008).

Star fruit (*Averrhoa bilimbi*; AB) is a natural ingredient that has potential to be used as a medicinal ingredient. The leaves, stems, and fruits of this plant have been studied for their ability to treat various diseases. AB contains antioxidants, namely alpha-tocopherol, and other active substances that have potential as antioxidants, including flavonoids, amino acids, proteins, purines, alkaloids, terpenes, and phenols (Krishnaiah et al., 2007). In addition, AB extracts show potency as anti-microbials (Norhana et al., 2009; Wahab et al., 2009), and AB leaf extracts may play a role in mediating BP in cats (Pérez-Vizcaíno et al., 2002). The aim of the present study was to evaluate the roles of AB extracts in increasing NO concentrations and vascular dilatation on hypertension-induced rats.

MATERIALS AND METHODS

Study design

This study was approved by the ethical committee of the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia (registration number: 2.KE.091.05.2019). The study was arranged using the Randomized Pretest-Posttest Control Group Design with single-blind administration.

Male Wistar rats (*Rattus norvegicus*) were obtained from the Laboratory Animal Research Center for Food and Nutrition, Gadjah Mada University, Yogyakarta, Indonesia. Rats were randomly selected from male rats 2~3 months of age and weighing 200~250 g reared under artificial induced-hypertension. Exclusion criteria included Wistar rat who had a physical disability or abnormality, or were unhealthy or pregnant. Rats were considered hypertensive if the systolic BP was >170 mmHg for two consecutive days after being induced with 30% ethanol for 15 days (Badyal et al., 2003).

Research procedures

Extracts were obtained from fresh bilimbi fruit (AB) which had been macerated with 96% ethanol in the Laboratory of Pharmacognosy and Phytochemical, Faculty of Pharmacy, Airlangga University. Hypertension was induced by oral administration of 30% ethanol for 15 days to obtain systolic pressure of 170~200 mmHg. The dose of AB extracts administered to rats was based on previous studies, which used 40 g/kg body weight (BW) (Alipin et al., 2018). BP was measured by the tail-cuff method and

was performed following the tail-cuff method using a Sphygmomanometers (Columbus Instruments, Columbus, OH, USA). NO was measured in blood serum using enzyme-linked immunosorbent assays with Total Nitrate Detection Kit Assay designs (R&D Systems, Inc., Minneapolis, MN, USA). Blood vessel histopathology was performed on the posterior vena cava stained with hematoxylin and eosin (H&E).

Data analysis

BP and NO concentrations before and after treatment were analyzed using paired-sample *t*-tests and independent *t*-tests. Posterior vena cava histopathology was analyzed descriptively using Image J (National Institutes of Health, Bethesda, MD, USA).

RESULTS AND DISCUSSION

Several active compounds were identified in the extracted AB. Results from thin layer chromatography (TLC) analysis indicated that the extract contained alkaloids, polyphenols, flavonoids, saponins, tannins, and terpenes. The TLC method is widely used to separate compounds with chemical reaction principle stationary phases and mobile phases using ultraviolet light to indicate specific color changes corresponding to different compounds (Fig. 1).

Rats were subjected to initial tests to measure baseline BP, for which all rats were in the normal range (110~130 mmHg). Hypertension was then induced by oral administration of 30% ethanol for 2 weeks. The average BP before and after ethanol treatment for all groups is presented in Table 1.

BP was similar in the P0 group before and after ethanol treatment ($P>0.05$). However, BP significant decreased ($P<0.05$) in all groups receiving AB extracts. Among the treatment group, lowest BP was observed for the P3 group (dose 40 mg/kg BW extract) (Table 1). Significant differences in NO concentrations were observed before and after treatment ($P=0.03$) with ethanol in P1, P2, and P3 (Fig. 2), with the highest increase in NO concentration observed for P2 (dose 20 mg/kg BW extract).

The posterior vena cava was stained with H&E for histopathological analysis after treatment with AB (Fig. 3).

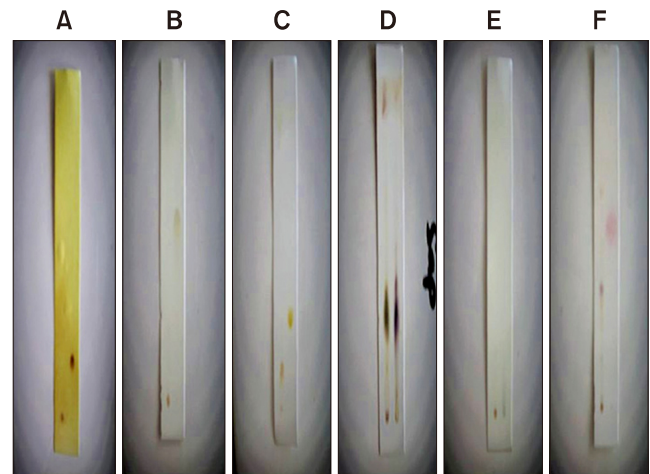


Fig. 1. The results of running thin layer chromatography from *Averrhoa bilimbi* L. extract showed positive for alkaloid (A), phenolic (B), flavonoid (C), saponin (D), tannin (E), and terpenes (F).

Cross-sections of the posterior vena cava showed no changes to the structures of the endothelial or tunica adventitia cell walls in the control group (Fig. 3A and Fig. 3B). However, in the induction group (Fig. 3C and Fig. 3D), the tunica adventitia wall endothelia was inflamed and contained necrotic cells, and the vacuolar was degenerated. In addition, in rats that received AB (Fig. 3E and Fig. 3F), posterior vena cava histopathological cross-sections showed dilatation and contraction of endothelial and tunica adventitia cells.

Several active compounds were identification in AB extracts by TLC, including flavonoids, saponins, polyphenols, essential oils, and anthraquinone (Fig. 1). This observation is similar with previous studies that reported that AB contains carotenoids, tannins, ascorbic acid, phenols, oxalic acid, flavonoids, saponins, triterpenoids, and polyphenols (De Lima et al., 2001; Galleano et al., 2010; Singh et al., 2012). Flavonoids were present in the qualitative content of AB extracts as active compounds. Ghasemi et al. (2009) reported that the quantitative content of flavonoids in *Citrus* sp. ranges from 0.30 to 31.10 mg/g of extract.

All rats had normal BP before treatment. After treatment with 30% ethanol for 2 weeks, all rats experienced a significant increase in systolic BP (Table 1). BP above 170 mmHg was categorized as hypertension. Once etha-

Table 1. Average blood pressure of rats in the different treatment groups

Treatments	Normal blood pressure (mmHg)	Pre-treatment blood pressure (mmHg)	Post-treatment blood pressure (mmHg)	Paired differences	Significance (2-tailed)
P0	127±2 ^a	192±3 ^a	189±3 ^a	75.5±44.6	0.001
P1	118±3 ^a	193±2 ^a	80±6 ^b		
P2	119±2 ^a	196±2 ^a	111±8 ^b		
P3	123±3 ^a	197±1 ^a	95±4 ^b		

Different letters (a,b) in the same column indicate a significant difference at $P<0.05$ ($n=6$).

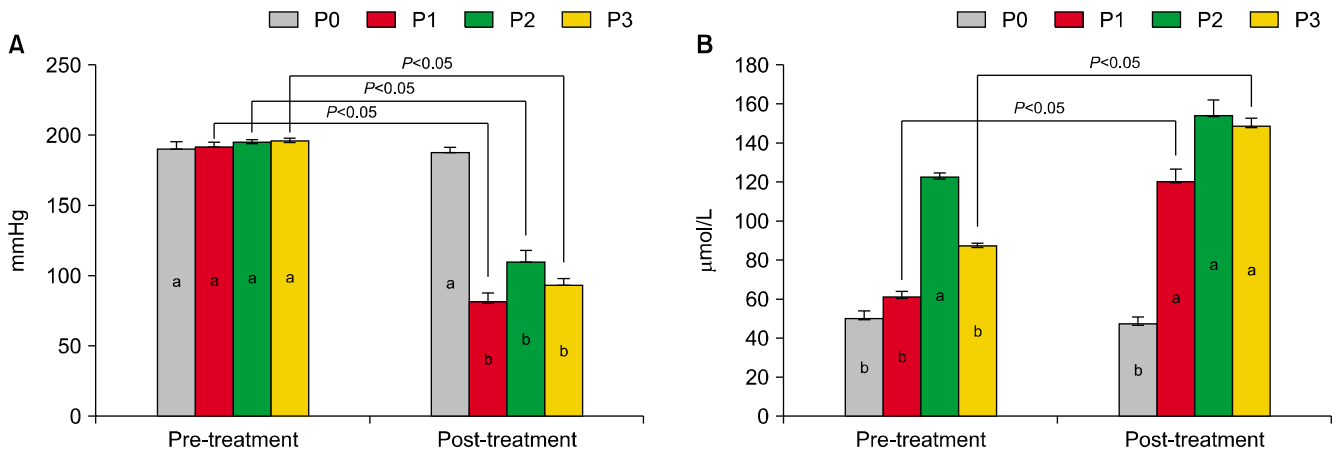


Fig. 2. Average blood pressure (A) and nitric oxide concentration (B) among treatment groups pre- and post-treatment with *Averrhoa bilimbi* L. extracts.

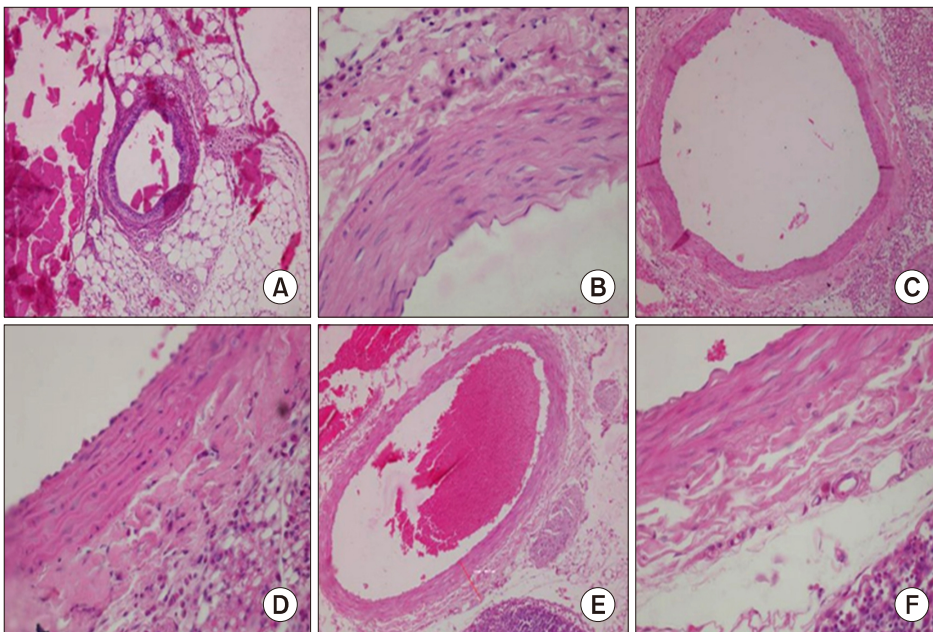


Fig. 3. Histopathological cross-section of the posterior vena (hematoxylin and eosin; 40 \times).

not is absorbed through the portal vein, it is immediately metabolized into acetaldehyde by the enzyme ethanol dehydrogenase, and is oxidized into acetate by acetaldehyde dehydrogenase/oxidase. Acetate induces formation of free radicals and lipid peroxidation that can damage cell membranes, leading to cardiovascular system dysfunction. Oxidative stress in the vascular system produces free radicals, inhibits formation of endothelial NOS (eNOS) and inhibits NO inactivation (Husain et al., 2004; Li et al., 2005).

BP in rats decreased significantly ($P < 0.05$) after treatment with AB extracts (P2 and P3 group) and captopril (P1) compared with the control group (P0) (Table 1). AB extracts may lower BP by flavonoids binding to nitrate peroxynitrite radical metabolites, which are derived from ethanol acetaldehyde free radicals in the blood. This study is consistent with results from López-López et al. (2004),

which reported that flavonoids can lower BP and repair endothelial dysfunction in experimental animals. In addition, Sánchez et al. (2006) suggested that diets containing flavonoids can lower BP and restore endothelial dysfunction by increasing eNOS, decreasing NADPH oxidase activity, and decreasing gene expression of *P47phox*. Furthermore, flavonoids exhibit antihypertensive roles, increase synthesis thromboxane, and inhibit glutathione peroxidase activity (Duarte et al., 2002) and lipid peroxidation (Bentz, 2009; Perez-Vizcaino et al., 2009).

In some studies, AB has been shown suppress pathogenic bacteria, such as *Salmonella* (Norhana et al., 2009), *Streptococcus*, *Clostridium* sp. (Zakaria et al., 2007), *Aeromonas*, *Escherichia coli*, *Klebsiella*, and *Bacillus subtilis* (Wahab et al., 2009). The active compounds in AB may further lower BP by oxidative modification of chemical reactions via acetaldehyde radical metabolites of ethanol, which

are easily eliminated, thereby preventing binding to NO. Krishnaiah et al. (2007) reported that the active compounds in flavonoids prevent oxidative free radicals function by acting antagonistically, direct inhibition, or by breaking down hydroperoxide to peroxy radicals. BP is known to be associated with the concentration of nitrate in the blood.

Our results showed that the decrease in BP was followed by an increase in NO concentration after treatment with *AB* extracts, indicating a direct link between BP and NO concentration in the blood. High nitrate concentrations are indicated by increased NO synthesis in the blood and activity of free NO molecules not binding to ROS. Plasma nitrite concentration is associated with the activity of enzyme eNOS (Kleinbongard et al., 2003).

NO is a fat-soluble molecule that plays many roles in activation of cellular reactions (signaling molecule). NO molecules function without the need for cell receptors and diffuse rapidly through cell membranes. Nitrate is secreted after the amino acid L-arginine is converted into L-citrulline via eNOS. The main mechanism of action of NO against target cells is via activating cellular enzyme guanylyl cyclase, which synthesizes cyclic guanosine monophosphate (cGMP). cGMP plays important roles in various cellular physiological processes (Tsoukias et al., 2004).

The increase NO synthesis indicated by high nitrate concentrations after treatment cannot be directly attributed to *AB* extracts. However, after observing a decline in BP treated rats, it can be presumed that the BP decrease exhibited in group P3 and P2 is followed by an increase in nitrate. In the control group (P0), the concentration of NO dropped slightly after treatment, and rats remained hypertensive with no change in nitrate concentration following administration of saline. Moreover, NO regulates contraction of vascular smooth muscle cells, which is inhibited by hemoglobin and myoglobin binding. Lim et al. (2008) suggested that nitrosothiol-NO is a regulator and activator of leukocytes in the circulatory system, and inhibits secretion of mast cells and scavenges radical oxidants during inflammation.

Our histopathology results showed that blood vessels in the cross-section of the posterior vena cava of ethanol-induced rats were inflamed in the tunica adventitia walls, and exhibited pyknosis in the endothelia, vacuolar degeneration, and necrotic cells were present in the tunica intimal. Changes to the dilatation and contraction of the blood vessel were also observed in the tunica adventitia of the vena cava posterior in rats that received *AB*, indicating repair and regeneration of blood vessels treated by ethanol (Fig. 3).

After treatment with *AB*, the blood vessels of the rats were wider with more dilated lumens that after ethanol treatment, which can also be associated with increased

NO concentration. These results are in line with those from a previous study, suggesting that NO concentration in blood positively influences the cardiovascular system. Therefore, NO in the blood stimulates vasodilatation of muscle walls in blood vessels, inhibits adhesion of inflammatory cells, and inhibits leukocyte thrombosis formation on blood vessel walls. These results are consistent with those from Allen et al. (2005), who reported that NO concentration in blood plasma is more stable by converting NO to nitrite to nitrate reactions. In the vascular system, NO has three important functions. NO mediates vasodilation, functions as an antithrombotic agent, and inhibits leukocyte cell adhesion, monocyte migration, and secretion of proinflammatory cytokines from endothelial cell walls.

Under normal conditions, NO secretion in blood vessels can prevent cardiovascular-related endothelial disorders. Crawford et al. (2004) stated that the occurrence of sepsis in acute inflammation and blood flow dysfunction and hypotension can damage NO molecules induced by septic red blood cells, resulting in vasodilation via increased levels of S-nitrosohemoglobin, inducible NOS, and blood nitrite. Captopril is an angiotensin blockade drug that inhibits secretion of renin during conversion of angiotensinogen into angiotensin-I and angiotensin II by angiotensin-converting enzyme. Captopril stimulates aldosterone secretion, which can induce increased water retention and inhibit sodium to prevent increases in BP (Badyal et al., 2003). This study revealed that captopril can maintain BP at a normal value. Krishnaiah et al. (2007) suggested that using natural products as medical treatments had multiple benefits since they had fewer toxic and allergic effects than synthetic drugs.

This study suggested that ethanolic extracts of *AB* significantly increased the concentration of NO and decreased BP in a rat model of hypertension. In addition, this study showed that *AB* extracts inhibit endothelial cell contraction and induce dilation of the lumen of the posterior vena cava.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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